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Purification and Characterization of Insulin and the C-Peptide of Proinsulin from Przewalski's Horse, Zebra, Rhino, and Tapir (Perissodactyla)

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Within the order Perissodactyla, the primary structure of insulin has been strongly conserved. Insulin from Przewalski's horse and the mountain zebra (suborder Hippomorpha) is the same as that from the domestic horse and differs from insulin from the white rhinoceros and mountain tapir (suborder Ceratomorpha) by a single substitution (Gly → Ser) at position 9 in the A-chain. A second molecular form of Przewalski's horse insulin isolated in this study was shown to represent the γ-ethyl ester of the Glu^{17} residue of the A-chain. This component was probably formed during the extraction of the pancreas with acidified ethanol. The amino acid sequence of the C-peptide of proinsulin has been less well conserved. Zebra C-peptide comprises 31 amino acid residues and differs from Przewalski's horse and domestic horse C-peptide by one substitution (Gln^{30} → Pro). Rhino C-peptide was isolated only in a truncated form corresponding to residues (1–23) of intact C-peptide. Its amino acid sequence contains three substitutions compared with the corresponding region of horse C-peptide. It is postulated that the substitution (Pro^{23} → Thr) renders rhino C-peptide more liable to proteolytic cleavage by a chymotrypsin-like enzyme than horse C-peptide. C-peptide could not be identified in the extract of tapir pancreas, suggesting that proteolytic degradation may have been more extensive than in the rhino. In contrast to the ox and pig (order Artiodactyla), there was no evidence for the expression of more than one proinsulin gene in the species of Perissodactyla examined.

Several studies have used comparisons of either the amino acid sequence of insulin and the C-peptide of proinsulin (Markussen and Volund, 1974; Blundell and Wood, 1975) or the nucleotide sequence of the preproinsulin gene (Perler *et al.*, 1980; Steiner *et al.*, 1985) to calculate rates of molecular evolution and to infer phylogenetic relationships between species. The taxonomic and phylogenetic interrelationships of Perissodactyla, or odd-toed ungulates, have been studied in detail using the classical techniques of morphological analysis and are well defined (Prothero and Schoch, 1989). These mammals, therefore, constitute a valuable group in which to compare conclusions derived from molecular and morphological analyses.

The extant Perissodactyla are classified into the suborder Hippomorpha, which includes the horses and zebras (superfamily Equoidea) and the suborder Ceratomorpha, which includes the rhinoceroses (superfamily Rhinocerotoidea) and tapirs (superfamily Tapiroidea). This study describes the structural characterization of insulin and C-peptide isolated from the pancreas of four Perissodactyls: Przewalski's horse (*Equus przewalskii*), the mountain zebra (*Equus zebra*), the mountain tapir (*Tapirus pinchaque*) and the white rhinoceros (*Ceratotherium simum*).

MATERIALS AND METHODS

Tissue extraction. Pancreata were obtained postmortem (up to 12 hr after death) from single adult fe-

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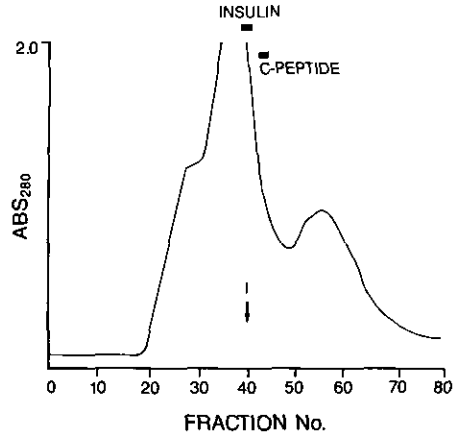


FIG. 1. Gel permeation chromatography on a Sephacryl S-100 column of an extract of pancreas from Przewalski's horse, after partial purification on Sep-Pak cartridges. The fractions denoted by the bars were used for the purification of insulin and C-peptide. The arrow shows the elution volume of pig insulin.

male specimens and were stored at −70°. Tissue (horse, 184 g; zebra, 245 g; rhino, 202 g; tapir, 149 g) was homogenized at 4° with 9 vol ethanol/0.7 *M* HCl (3:1 v/v) using a Waring blender. The homogenates were stirred for 16 hr at 6°. Peptides were isolated from the extracts using Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) as described (Henry *et al.*, 1991). Bound material was eluted with acetonitrile/water/trifluoroacetic acid (80.0:19.9:0.1) and lyophilized (Savant Speed Vac).

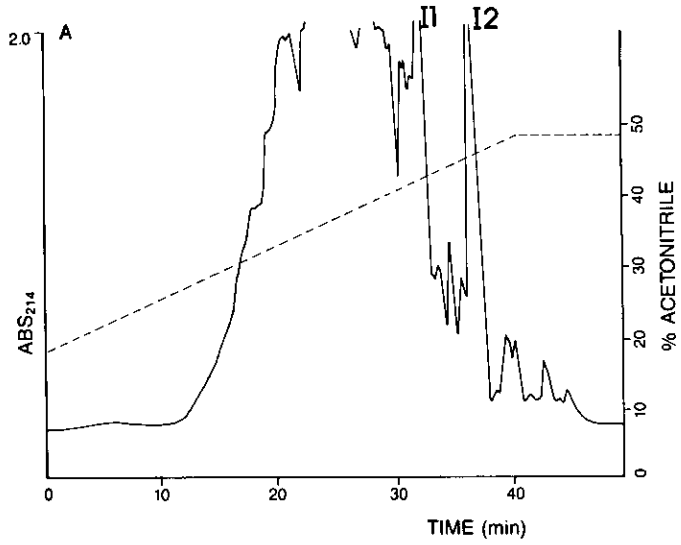
Radioimmunoassay procedure. Insulin-like immunoreactivity was measured by radioimmunoassay using an antiserum raised against pig insulin in a procedure that has been described previously (Flatt and Bailey, 1981).

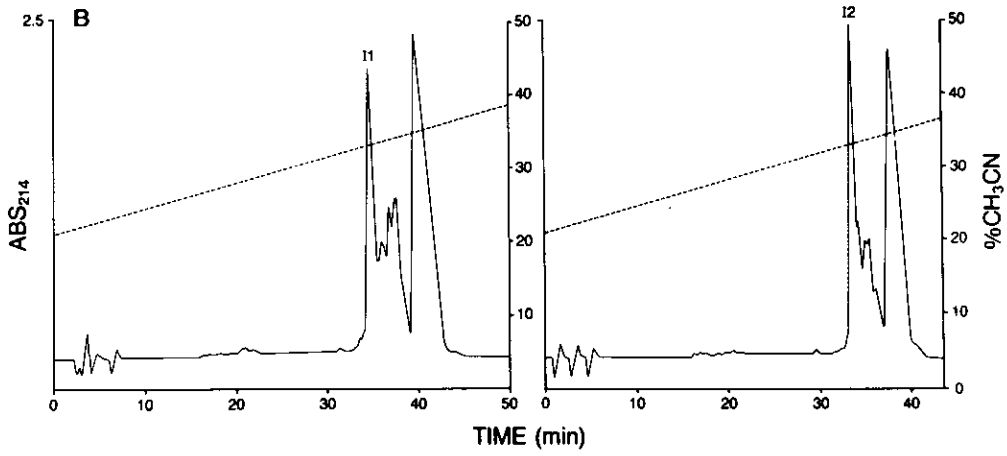
Purification of insulin. The same procedure was used for the purification of insulin and C-peptide from each species and so only the method used for the isolation of the peptides from Przewalski's horse is described in detail. The extract, after partial purification on Sep-Pak cartridges, was redissolved in 1% (v/v) trifluoroacetic acid (10 ml) and 50% of the total material was chromatographed on a 5 × 100-cm Sephacryl S-100 gel permeation column (Pharmacia-LKB, Uppsala, Sweden) equilibrated with 1 *M* acetic acid at a flow rate of 120 ml/hr. Fractions (10 ml) were collected and assayed for insulin-like immunoreactivity. The fraction containing maximum immunoreactivity was injected onto a 1 × 25-cm Vydac 218TP510 C-18 reversed-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1) at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 49% (v/v) over 40 min using a linear gradient. Absorbance was measured at 214 and 280 nm and individual peaks were collected by hand. Insulin-like immunoreactivity was measured at a dilution of 1:100. The fractions containing immunoreactivity were separately rechromatographed on a 1 × 25-cm Ultrapore 6RPSC288 C-3 reversed-phase HPLC column (Beckman, Duarte, CA) equilibrated with acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1) at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 38% (v/v) over 50 min using a linear gradient. Insulins were purified to apparent homogeneity by chromatography on a 0.46 × 25-cm Vydac 214TP54 C-4 reversed-phase HPLC column using the same elution conditions as in the previous chromatography.

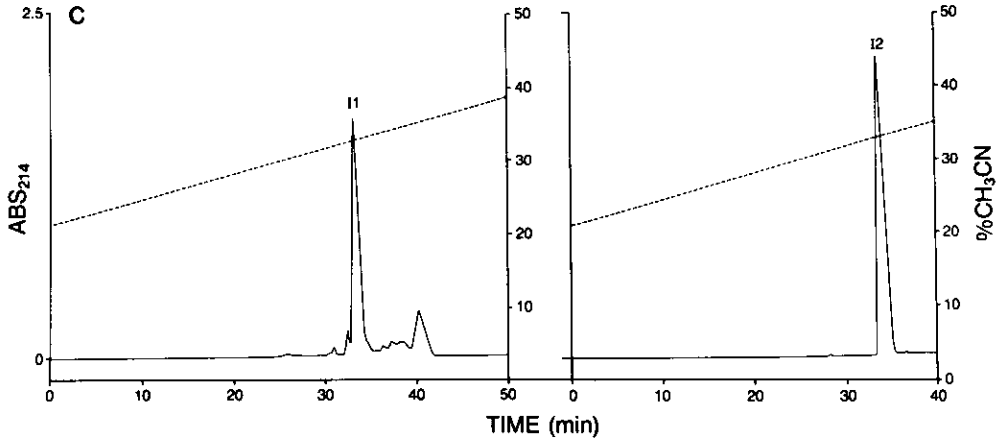
Purification of C-peptide. The fractions from gel permeation chromatography containing C-peptide (denoted by the bar in Fig. 1) were injected onto a 1 × 25-cm Vydac 218TP510 C-18 HPLC column under the same conditions used for the purification of insulin. C-peptide was purified to apparent homogeneity by chromatography on a 0.46 × 25-cm Vydac 214TP54 C-4 HPLC column equilibrated with acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 42% (v/v) over 50 min.

Structural characterization. Insulins (5–10 nmol) were reduced (with dithiothreitol) and pyridylethylated (with 4-vinylpyridine) as previously described (Conlon and Hicks, 1990). The derivatized A-chain and B-chain were separated by reversed-phase HPLC on a Vydac C-18 column using the elution conditions shown in Fig. 2. Amino acid compositions were determined by precolumn derivatization with phenylisothiocyanate using an Applied Biosystems Model 420A derivatizer and Model 130A separation system as previously described (Conlon and Hicks, 1990). Hydrolysis (6 *M* HCl for 24 hr at 110°) of approximately 1 nmol of sample was carried out. The detection limit for phenythiocarbamyl amino acids was 1 pmol. Automated Edman degradation was performed using an Applied Biosystems Model 471A sequenator modified for on-line detection of phenylthiohydantoin amino acids under gradient elution conditions. The detection limit for the PTH derivatives was 0.5 pmol.

FIG. 2. Reversed-phase HPLC on (A) Vydac C-18, (B) Ultrapore C-3, and (C) Vydac C-4 columns of insulin from an extract of Przewalski's horse pancreas, after partial purification by gel permeation chromatography. The peaks denoted by I1 and I2 contained insulin-like immunoreactivity. The dashed line shows the concentration of acetonitrile in the eluting solvent.


A
I1
I2
2.0
ABS214
% ACETONITRILE
TIME (min)


B
I1
I2
2.5
ABS214
%CH3CN
TIME (min)


C
I1
I2
2.5
ABS214
%CH3CN
TIME (min)

RESULTS

Purification of Insulins from Przewalski's Horse

The elution profile on a Sephacryl S-100 gel permeation of the extract of horse pancreas is shown in Fig. 1. Insulin-like immunoreactivity was eluted from the column as a single peak, with the same elution volume as pig insulin. The fraction denoted by the bar contained maximum immunoreactivity and was chromatographed on a semi-preparative C-18 reversed-phase column (Fig. 2A). Insulin-like immunoreactivity was associated with the two prominent peaks designated I1 and I2. These peaks were separately chromatographed on a semi-preparative C-3 column (Fig. 2B) and the immunoreactive peptides were purified to apparent homogeneity on an analytical Vydac C-4 column (Fig. 2C). The yield of purified insulin I1 was 13 nmol and the yield of insulin I2 was 11 nmol.

Purification of Insulin from the Zebra, Rhinoceros, and Tapir

Insulin was isolated from pancreatic extracts from the zebra, rhino, and tapir under the same conditions of chromatography used for the purification of Przewalski's horse insulin. In each case, the antisera used in radioimmunoassay detected two molecular forms of insulin but only the earlier eluting, major component (representing the unmodified peptide) was purified to homogeneity. The approximate final yields of pure peptides were zebra, 14 nmol; rhinoceros, 11 nmol; and tapir, 6 nmol. As the tissues were collected at varying times postmortem, these amounts probably do not accurately reflect the actual concentrations in the functioning pancreas.

Purification of C-Peptide from Przewalski's Horse

A radioimmunoassay for C-peptide was not in the operation in the laboratory. Consequently, fractions from the Sephacryl S-100 column corresponding to the elution volume of peptides in molecular mass zone 2000–5000 were systematically subjected to HPLC and screened for the presence of C-peptide by measuring absorbance at 214 nm. Detection of C-peptide was facilitated by the fact that mammalian C-peptides are generally more hydrophilic (lower retention time) than insulin and do not contain either tyrosine or tryptophan residues and so do not show absorbance at 280 nm. The fraction denoted by the bar (Fig. 1) was injected onto a semi-preparative C-18 reversed-phase HPLC column (Fig. 3A) and the well defined, early eluting peak designated CP was subsequently shown to contain C-peptide. Przewalski's horse C-peptide was purified to apparent homogeneity on an analytical Vydac C-4 column (Fig. 3B) and the yield of pure material was 9 nmol.

Purification of C-peptide from Zebra and Rhino

C-peptide was isolated from the extracts of zebra and rhino pancreas using the same strategy employed for the purification of horse C-peptide. The yields of pure material were zebra, 10 nmol; and rhino, 5 nmol. Attempts to purify tapir C-peptide were unsuccessful. No peptide with similar elution volumes on Sephacryl S-100 and retention times on reversed-phase HPLC as C-peptide from the other species of Perissodactyla was identified in the extract of tapir pancreas.

Structural Characterization

The amino acid compositions of the proinsulin-derived peptides isolated in this study are shown in Table 1. The data indicate that the structures of the Perissodactyla insulins are very similar. The composition of the B-chain of insulin was the same for the four species. The A-chain Przewalski's horse insulin was same as that of the zebra and differed from that of the rhino

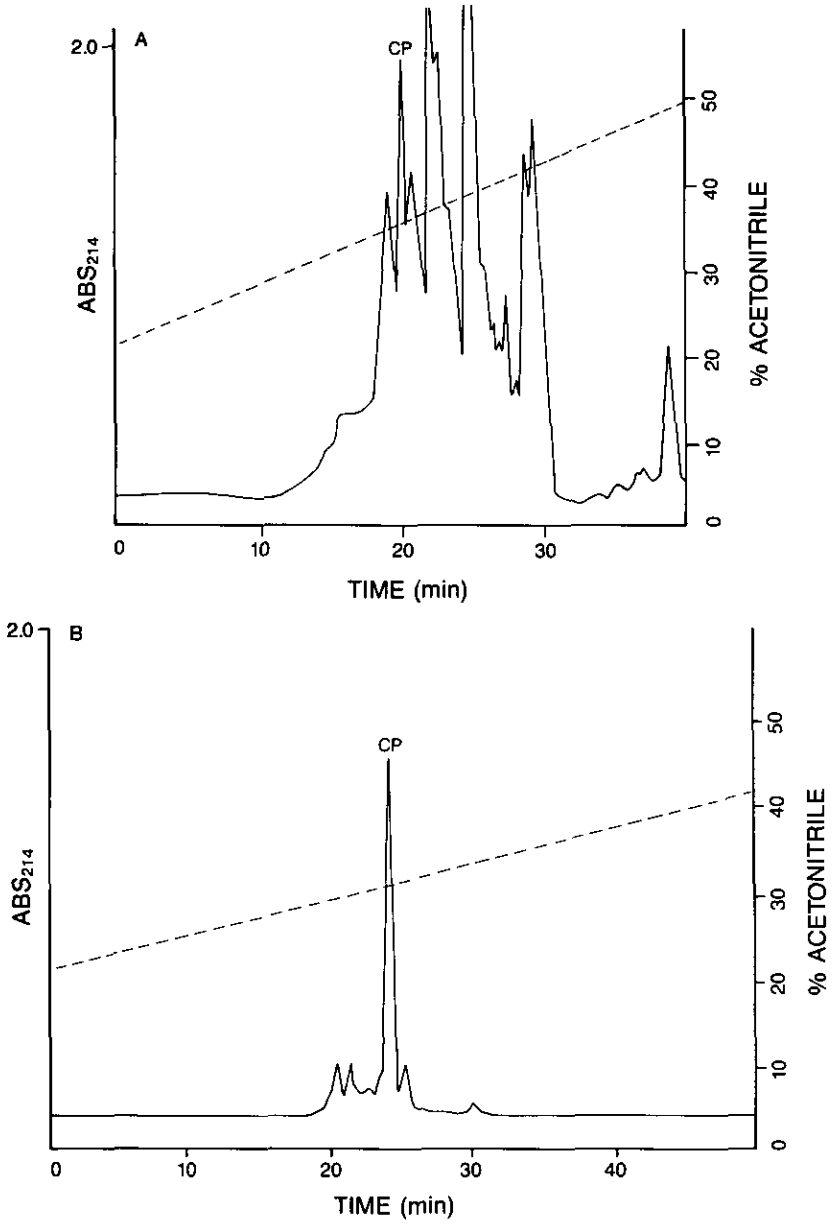


FIG. 3. Reversed-phase HPLC on (A) Vydac C-18 and (B) Vydac C-4 columns of the C-peptide of proinsulin from an extract of Przewalski's horse pancreas, after partial purification by gel permeation chromatography. The peak denoted by CP contained the C-peptide.

and tapir by the substitution of a glycine by a serine residue. The composition of both the A-chain and the B-chain of the two molecular forms of Przewalski's horse insulin (I1 and I2) were the same. The complete primary structures of the pyridylethylated derivatives of the A-chains and B-chains of the four insulins were determined by automated Edman degradation and the results of the sequence analysis are summarized in Table 2. Przewalski's horse (P. horse) insulin has the same structure as insulin from

TABLE 1
AMINO ACID COMPOSITION OF INSULIN AND THE C-PEPTIDE OF PROINSULIN FROM PRZEWALSKI'S (P.) HORSE, ZEBRA, RHINO, AND TAPIR

	Residues/mol peptide				
Amino acid	P. horse I1	P. horse I2	Zebra	Rhino	Tapir
		A-Chain			
Asx	1.8 (2)	2.1 (2)	1.9 (2)	2.0 (2)	2.2 (2)
Glx	4.0 (4)	4.2 (4)	3.9 (4)	4.0 (4)	4.3 (4)
Ser	1.0 (1)	1.0 (1)	1.1 (1)	1.9 (2)	2.0 (2)
Gly	2.0 (2)	1.9 (2)	2.2 (2)	1.1 (1)	1.3 (1)
Thr	1.2 (1)	1.0 (1)	1.1 (1)	0.9 (1)	0.7 (1)
Tyr	2.1 (2)	2.0 (2)	1.7 (2)	1.8 (2)	1.7 (2)
Val	0.4 (1)	0.4 (1)	0.5 (1)	0.5 (1)	0.5 (1)
Ile	1.4 (2)	1.5 (2)	1.4 (2)	1.5 (2)	1.5 (2)
Leu	2.3 (2)	2.2 (2)	2.0 (2)	2.1 (2)	2.2 (2)
		B-Chain			
Asx	1.0 (1)	1.0 (1)	1.1 (1)	1.1 (1)	1.0 (1)
Glx	2.8 (3)	3.0 (3)	3.0 (3)	2.9 (3)	3.0 (3)
Ser	0.9 (1)	0.9 (1)	1.0 (1)	1.0 (1)	0.9 (1)
Gly	2.7 (3)	3.0 (3)	3.0 (3)	3.0 (3)	3.0 (3)
His	1.8 (2)	1.9 (2)	1.8 (2)	1.7 (2)	1.8 (2)
Arg	1.0 (1)	1.1 (1)	1.2 (1)	1.1 (1)	1.2 (1)
Thr	0.8 (1)	0.8 (1)	0.9 (1)	0.8 (1)	0.7 (1)
Ala	1.8 (2)	2.0 (2)	2.0 (2)	1.9 (2)	1.9 (2)
Pro	0.9 (1)	1.0 (1)	1.1 (1)	1.0 (1)	1.0 (1)
Tyr	1.7 (2)	1.8 (2)	1.7 (2)	1.8 (2)	1.7 (2)
Val	2.8 (3)	2.9 (3)	3.0 (3)	2.8 (3)	2.7 (3)
Leu	3.7 (4)	3.8 (4)	3.8 (4)	3.7 (4)	3.8 (4)
Phe	2.8 (3)	2.8 (3)	2.9 (3)	3.0 (3)	2.9 (3)
Lys	1.0 (1)	1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)
		C-Peptide			
Asx	0.9 (1)		1.1 (1)	1.0 (1)	
Glx	7.8 (8)		6.9 (7)	4.8 (5)	
Gly	8.4 (8)		8.0 (8)	7.0 (7)	
Thr				1.2 (1)	
Ala	3.3 (3)		3.2 (3)	2.2 (2)	
Pro	4.2 (4)		5.0 (5)	2.2 (2)	
Val	2.0 (2)		2.1 (2)	2.1 (2)	
Leu	4.7 (5)		4.9 (5)	3.1 (3)	

Note. The values in parentheses show the number of residues predicted from the proposed sequences.

the zebra and domestic horse (Harris *et al.*, 1956). Rhino insulin is the same as insulin from the tapir and the pig (Chance *et al.*, 1968). This conclusion was substantiated by the observation that a mixture of rhino, tapir, and pig insulin was eluted from a C-4 reversed-phase HPLC column, under the elution conditions shown in Fig. 2C, as a single sharp peak.

Edman degradation of the pyridylethylated derivative of the B-chain of P. horse insulins I1 and I2 gave identical results and both peptides coeluted from a reversed-phase HPLC column as a single peak. However, when the A-chain of insulin I2 was subjected to Edman degradation the yield of the phenylthiohydantion derivative of glutamic acid during cycle 17 was low and an additional peak, with a retention time the same as that of the phenylthiohy-

TABLE 2
A COMPARISON OF THE PRIMARY STRUCTURES OF INSULIN AND THE C-PEPTIDE OF PROINSULIN FROM SOME SPECIES OF UNGULATES

Species	Structure						
	A-Chain						
P. horse	GIVEQ	CCTGI	CSLYQ	LENYC	N		
Zebra	-----	-----	-----	-----	-		
Rhino	-----	---S-	-----	-----	-		
Tapir	-----	---S-	-----	-----	-		
Pig	-----	---S-	-----	-----	-		
Ox	-----	--ASV	-----	-----	-		
	B-Chain						
P. horse	FVNQH	LCGSH	LVEAL	YLVCG	ERGFF	YTPKA	
Zebra	-----	-----	-----	-----	-----	-----	
Rhino	-----	-----	-----	-----	-----	-----	
Tapir	-----	-----	-----	-----	-----	-----	
Pig	-----	-----	-----	-----	-----	-----	
Ox	-----	-----	-----	-----	-----	-----	
	C-Peptide						
P. horse	EAEDP	QVGEV	ELGGG	PGLGG	LQPLA	LAGPQ	Q
Zebra	-----	-----	-----	-----	-----	----P	-
Rhino	-----	-----	-----	--A--	-LT		
Pig I	---N-	-A-A-	-----	L-*-*	--A--	-E--P	-
Pig II	---N-	-*-A-	-----	L-*-*	--A--	-E--P	-
Ox I	-V-G-	---AL	--A--	--A--	-****	*E--P	-
Ox II	-V-G-	---AL	--A--	L-A--	-****	*E--P	-

Note. A dash denotes conservation and an asterisk denotes deletion of amino acid residues.

dantoin derivative of glutamic acid γ-ethyl ester, (Sigma, St. Louis, MO) was observed. This peak was not seen during sequence analysis of insulin I1. It is concluded that P. horse insulin I2 represents an artifactually modified form of insulin probably formed during extraction of the pancreatic tissue with acidified ethanol.

The amino acid compositions of the purified C-peptides of the Perissodactyla proinsulins indicate that this region of the molecule has been less well conserved than that of insulin (Table 1). The data show that P. horse C-peptide contains a total of 31 amino acid residues and differs from zebra insulin by the substitution of a glutamic acid/glutamine residue by proline. The composition of rhino insulin, however, indicates a total of only 23 amino acid residues. The results of automated Edman degradation are shown in Table 3. The primary structure of Przewalski's horse C-peptide is the same as that of C-peptide from the domestic horse (Tager and Steiner, 1972) and differs from that of zebra C-peptide by the substitution (Gln → Pro) at position 30. The data confirm that rhino C-peptide comprises only 23 amino acid residues but a comparison of its sequence with those of other C-peptides from ungulate species (Table 2) suggests that the peptide isolated in this study represents an NH_2-terminal fragment of a larger C-peptide.

DISCUSSION

The present study complements earlier related work in which pancreatic polypep-

TABLE 3
AUTOMATED EDMAN DEGRADATION OF THE C-PEPTIDE OF PROINSULIN FROM PRZEWALSKI'S HORSE, ZEBRA, AND RHINO

Cycle number	P. horse		Zebra		Rhino	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Glu	5050	Glu	5640	Glu	2782
2	Ala	6836	Ala	6705	Ala	2975
3	Glu	4285	Glu	5033	Glu	1672
4	Asp	2908	Asp	3238	Asp	1080
5	Pro	4017	Pro	5441	Pro	1494
6	Gln	3123	Gln	4060	Gln	1575
7	Val	3374	Val	5141	Val	1552
8	Gly	3112	Gly	4283	Gly	1315
9	Glu	2239	Glu	3045	Glu	927
10	Val	2850	Val	4484	Val	1296
11	Glu	2039	Glu	2622	Glu	837
12	Leu	2562	Leu	3890	Leu	936
13	Gly	2650	Gly	3486	Gly	1055
14	Gly	3131	Gly	4283	Gly	1401
15	Gly	3135	Gly	4369	Gly	1464
16	Pro	2177	Pro	3369	Pro	813
17	Gly	2308	Gly	3498	Gly	1034
18	Leu	2180	Leu	3347	Ala	968
19	Gly	2005	Gly	3221	Gly	897
20	Gly	2579	Gly	3706	Gly	947
21	Leu	1792	Leu	2969	Leu	353
22	Gln	1802	Gln	2104	Leu	413
23	Pro	1268	Pro	1916	Thr	54
24	Leu	1312	Leu	1918		
25	Ala	1384	Ala	1951		
26	Leu	1244	Leu	1782		
27	Ala	1408	Ala	1743		
28	Gly	886	Gly	922		
29	Pro	616	Pro	592		
30	Gln	599	Pro	602		
31	Gln	613	Gln	249		

tide was isolated from the same four species of Perissodactyls (Henry *et al.*, 1991) but the conclusions to be drawn from a comparison of the amino acid sequences of the proinsulin-related peptides and the pancreatic polypeptides are different. Consistent with the accepted taxonomic classification, the structure of Przewalski's horse and zebra (suborder Hippomorpha) insulins are the same and insulins from the rhino and tapir (suborder Ceratomorpha) are the same. In contrast, tapir pancreatic polypeptide was more similar in structure to pancreatic polypeptide from the horse and zebra than to the rhino. Thus, the data from the present study support the hypothesis that extant tapirs and rhinoceroses evolved from a common ancestor that was distinct from the common ancestor of the horses and zebras (Prothero and Schoch, 1989) whereas the earlier study of Henry *et al.* (1991) did not. In general, the rate of molecular evolution of C-peptide has been greater than that of insulin (Markussen and Volund, 1974; Steiner *et al.*, 1985). Consistent with this observation, the C-peptide of

zebra proinsulin shows one amino acid substitution compared with horse C-peptide but both these molecules show three substitutions compared with the truncated form of rhino C-peptide isolated in this study (Table 2).

Examination of commercially available samples of bovine (Frank *et al.*, 1984) and porcine (Snel and Damgaard, 1988) proinsulin has led to the isolation of two distinct gene products in these species of Artiodactyla. The variant of bovine proinsulin contained the substitution (Pro → Leu) at position 16 in the C-peptide region and the second form of porcine proinsulin contained a deletion of the Ala residue at position 7 in the C-peptide region (Table 2). Although two molecular forms of insulin (I1 and I2) were isolated from the extract of horse pancreas, subsequent structural characterization showed that the second form was probably an artifact of the extraction procedure. We confirmed that overnight incubation of porcine insulin with acidified ethanol led to the formation of the γ-ethyl ester of the A17 glutamic acid residue together with several other unidentified degradation products. Porcine insulin was, however, completely stable in this solvent for 1 hr at 0° and so we recommend that, if acidified ethanol is used to extract pancreatic tissue, temperatures must be kept low and extraction times kept short. In this study, we found no evidence for C-peptide heterogeneity suggesting that the putative duplication that led to two proinsulin genes in the pig and ox may have taken place after the time of divergence of the Artiodactyla and Perissodactyla.

The isolation of rhino C-peptide in a truncated molecular form with 23 amino acid residues is consistent with studies in other species. Rat C-peptides I and II are subject to cleavage by a chymotrypsin-like enzyme at the site of the Gln^{22}–Thr^{23} bond (Tager *et al.*, 1973) and evidence for processing of ratfish proinsulin within the C-peptide region by a chymotrypsin-like enzyme has been obtained (Conlon *et al.*, 1989). An analysis of the sites of cleavage by α-chymotrypsin of 235 protein substrates catalogued in the Proteolysis Data Bank (Institut Pasteur, Paris, France) has shown that cleavage of peptide bonds incorporating a proline residue are extremely rare (Keil, 1987). It is suggested, therefore, that the increased susceptibility of rhino C-peptide to intracellular proteolysis compared with horse and zebra C-peptide is a consequence of the substitution (Pro^{23} → Thr). Similarly, the inability to identify C-peptide in the extract of tapir pancreas may be a consequence of the fact that the molecule may be processed at more than one site to generate smaller fragments which would not have been detected by the methods used in this study. C-peptide was also isolated from the pancreas of the dog (Kwok *et al.*, 1983) and fox (Majewski *et al.*, 1987) as a 23-residue fragment but, in these two cases, a trypsin-like proteolytic cleavage occurred at site of the Arg^8–Asp^9 bond.

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